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Exercise training enhances resistance to infection and boosts immune responsiveness to influenza in aged mice

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Exercise training enhances resistance to infection and boosts immune responsiveness to influenza in aged mice

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Signatures have been redacted for privacy

For my parents
who always taught me
to reach for the stars

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Introduction

Influenza is an acute viral respiratory infection that may result in high morbidity and mortality. Worldwide pandemics occur when influenza virus undergoes antigentic shift, which involves a reassortment of the segmented RNA genome of the influenza virus or related animal influenza virus in a animal host. The reassortment of the genome may result in new hemagglutinin and/or neuraminidase glycoproteins on the viral surface (Janeway et al., 2001). The new virus may be poorly recognized, if at all, by antibodies and T cells in the human host directed against the original virus. Thus, most people are highly susceptible to the new virus, and severe infection results (Janeway et al., 2001). In the United States 40,000 – 80,000 deaths occur each year from influenza infection (Smith et al., 1992). A greater number of deaths may occur when the virus has undergone antigenic shift. Approximately 80-90% of the deaths from influenza occur in the elderly.

Influenza vaccination is a critical tool for the prevention of the disease. However, vaccination is less effective in older adults. The influenza vaccine is only 30-60% effective in preventing clinical illness among elderly individuals as compared to 70-90% effective in young adults (Gross et al, 1999; Demicheli et al., 2000).

Immune function is compromised in the aged. That age-related decline of immunity may result in poorer vaccine efficacy. The ability to respond effectively when an infection occurs also is reduced (Pawelec et al., 1999). The elderly tend to have more severe disease symptoms during infectious episodes, which may result in higher in mortality rates (Garg et al., 1996). Among older adults, influenza virus infection may be compounded by secondary bacterial pneumonia, leading to higher rates of mortality (Louria et al., 1959).

The influenza A virus is highly contagious. It preferentially replicates in the epithelial cell layers lining the airway of the respiratory tract, but also can infect macrophages and other leukocytes. Most cell types carry the influenza virus receptor, which is a sialic acid containing cell surface glycoprotein (Janeway et al., 2001). Productive influenza virus infection in epithelial cells leads to the destruction of host cell pre-mRNAs, translational inhibition of cellular mRNAs, and death of the host cell by cytolytic or apoptotic mechanisms (Julkunen et al., 2001).

The immune system can respond in many ways to restrict the spread of the virus. Cytokines recruit inflammatory cells to the site of infection and activate the host antiviral defense system. Th1 cytokines (IL-2, IFN-γ, IL-12 and TNF-α) drive cell-mediated immune responses, whereas Th2 cytokines (IL-4, IL-5 and IL-10) are important for B cell growth and differentiation and drive humoral immune responses (Shearer et al., 1997). Other defenses include activation of apoptotic pathways and antigen presentation by macrophages and dendritic cells (Julkunen et al., 2001).

Several therapies may help improve immune function with advancing age such as vitamins, anti-oxidants, hormones, dietary restriction, and exercise. Regular exercise has been shown to have mental, physical, physiological, and immunological benefits (Hirokowa et al., 1997). In addition to positive effects on immune function, exercise decreases the risk of developing heart disease, type II diabetes, osteoporosis, arteriosclerosis, and some types of cancer (Mazzeo et al., 1998). Specifically, exercise is now known to have effects on immune cells and their function (Woods et al., 2003). Exercise may prevent immunosenescence by promoting immune improvement. One 12-week moderate exercise study performed on older adult women showed that exercise increased natural killer (NK) activity and T cell function

in previously sedentary subjects (Nieman et al., 1993). Another study that compared sedentary controls to highly conditioned endurance-trained females (average age 73) found T cell function and NK activity was higher in exercise trained individuals (Pedersen et al., 2000). A study by Guelder et al. showed that T cells from older active women who have performed long-term moderate exercise exhibited higher responsiveness (CD25 expression) to stimulation *in vitro* than T cells from a control group of sedentary subjects. Endurance training in older males was associated with increased T cell function and cytokine production suggesting an attenuation of the age related immune function decline (Shinkai et al., 1995). An experiment on aged mice found that exercise training restored the percentages of naïve and memory cells in the spleen toward that of young mice (Woods et al., 2003). Exercise training may have induced apoptosis/necrosis of memory T cells, reduced production of memory cells, or cause a reversion of $CD44^{hi}$ expressing cells back to $CD44^{lo}$ expression. Kohut et al. (2001) found that exercise was associated with an enhanced production of the viral specific Th1-associated cytokines IL-2 and IFN-γ in aged mice. To our knowledge, it has not yet been demonstrated whether exercise is effective in improving resistance to infection among an aged population, and whether any exercise-induced improvement in resistance to infection is associated with corresponding enhancement of specific antiviral immune defenses.

The purpose of this investigation was to determine if regular moderate exercise training alters immune response to infection with influenza in aged mice. The hypothesis of this thesis is that exercise will enhance the immune response to influenza virus infection, as evidenced by increased CD80/86 expression and by up-regulation of cytokine production in

both the spleen and mediastinal lymph nodes, resulting in attenuated disease and viral load in experimentally infected mice.

Literature Review

The elderly population is more susceptible to influenza and has a higher death rate than young individuals. An understanding of age related changes in immune function and disease susceptibility is necessary to understand the underlying causes of this phenomenon.

Aging and immunity overview

Immune senescence is the gradual deterioration in immune function that occurs with aging. It is manifested as reduced immune responsiveness measured *in vitro* and as impaired *in vivo* immune response to acute infection and vaccination. This age related decline is largely due to diminished T cell function that adversely affects proliferation, cytokine production, and signal transduction (Pawelec et al., 1999). The elderly have an age-related decrease in naïve T cells and an increase of memory cells. This shift from naïve to memory cells causes a reduced ability to mount a response to new antigens (Gardner et al., 2002). Thus, there is an increased risk and severity of infection from new pathogens, such as influenza. In addition, the higher percentages of memory cells that are present have a significantly reduced ability to recall and respond to previous antigens (Ernst et al., 1993). In addition, declining host immune responses, particularly cellular immunity, account for the increased susceptibility to influenza virus infection of the aged. Aged mice have been shown to have decreased cytotoxic T lymphocyte (CTL) activity against virally infected targets, reduced proliferative responses, lower interleukin-2 production, defective antigen presentation, and a decreased numbers of splenic precursor cells (Bender et al., 1995). B cells and macrophages also function less effectively in the aged (Doppler et al., 1994). Other studies have shown diminished natural killer cell (NK) activity in aged individuals compared to young individuals (Fiatarone et al., 1989). Increased susceptibility to influenza infection

has also been associated with the impaired immune function of T helper 1 cells (Kostense et al., 1998)

In the innate immune system the functions of NK cells, macrophages, and neutrophils, decreased with aging (Ginaldi et al., 1999). In the aged mouse, alveolar macrophages are decreased in number and are not as efficient at presenting antigen to T cells. In addition, more macrophages are needed to effectively activate T cells (Bender et al., 1995). In addition, neutrophils have impaired chemotaxis, degranulation, and phagocytosis (Wenisch et al., 2000). Thus, both the adaptive and innate immune factors decline with age.

Innate immune defense and aging

Macrophages, NK cells, and neutrophils provide the first line of defense against bacterial and viral infections. Decline in their function may result in increased incidences of bacterial and viral pneumonia and gastrointestinal and skin infections in the aged (Gavazzi et al., 2002). In aged mice, splenic and activated peritoneal macrophages express lower levels of all toll-like receptors and secrete lower amounts of IL-6 and TNF-α when stimulated (Dobber et al., 1995). Thus, aging can cause impaired innate defense.

Shift in naïve/memory population

There is an age-related decrease of naïve T cells and an increase of memory cells. With increasing age there are fewer naïve T cells available to respond to new pathogens and an increase in the number of memory $CD8^{+}28^{-}57^{+}$ T cells (Effros et al., 2000). Age-associated alterations in naïve $CD8^{+}$ cell function are not found after primary stimulation, but may become apparent upon restimulation. This shift from naïve to memory cells also causes a shift in the cytokine environment (Timm et al., 1999).

Cytotoxic T cells and aging

Cytotoxic T lymphocyte (CTL) activity in general and anti-influenza CTL activity decrease with age (Fagnoni et al., 2000). Previous studies by Bender et al. (1991) have shown that CTL activity is critical for recovery from influenza infection. It has been shown that influenza infected nude mice do not clear the infection and shed influenza virus from their lungs indefinitely. However, adoptive transfer of CTL to the infected nude mice leads to viral clearance from the lungs (Bender et al., 1991). This again suggest that CTL's are important in clearance of viral infection. Administration of anti-influenza antibody to nude mice will temporally halt viral shedding as long as the antibody is present. Thus, antibody neutralizes the virus and halts it rapid replication but does not completely destroy it. Aged mice have a prolonged influenza infection that correlates with lower anti-influenza CTL activity, as well as prolonged pulmonary viral shedding (Bender et al, 1993). The cellular and molecular basis of the age-related decline in CTL activity have been the subject of several studies. Some suggest that with increasing age there is only a slight decline in responsiveness of the individual T cell, but a large decline in the number of cells that can respond (Bender et al, 1993). It has been demonstrated that aged mice have a lower precursor anti-influenza CTL frequency (Effros et al., 1983). Therefore, it appears that CTL response to influenza infection is impaired with aging, and this may be due in part, to a lower frequency of CTL specific cells.

Aging and the Th1/Th2 cytokine balance

Since the discovery of T helper cell subsets, there have been studies on whether aging leads to shifts in the Th1/Th2 response. There are three functional subsets of CD4+ T cells. T helper 1 cells (Th1) secrete IL-2, IFN-γ, IL-12 and lymphotoxins, which induce cellular

immunity against intracellular bacteria and parasites. T helper 2 (Th2) cells secrete IL-4, IL-5, IL-10, and IL-13 and promote a humoral response against extracellular pathogens (Gardner et al, 2002). Most the circulating CD4+ cells are T helper 0, which produce both Th1 and Th2 cytokines (Janeway et al., 2001).

During aging, phenotypical and functional changes are seen within murine CD4 T cell compartments. Phenotypically, there is a shift from naïve CD4+ T cells to memory cells. This change is associated with a decrease of IL-2 production and an increase of IL-4 and IFN-γ production (Dobber et al., 1995).

The ability of B cells to produce particular immunoglobin isotypes depends on cytokines produced by CD4 T cells (Dobber et al., 1995). CD4 T cells are an important source of cytokines. Thus, changes in CD4 T cell populations that occur during aging may be responsible for changes in humoral immunity (Dobber et al, 1995).

The same phenomenon is observed in humans. IL-4 and IFN-γ production by CD4 T cells increases during aging and IL-2 production declines with advancing age. These age-associated changes in the cytokine balance may result in altered response to influenza vaccine and increased susceptibility to influenza (Dobber et al., 1995).

Immune defense against influenza virus

Influenza and IFNs

Interferons (IFNs) protect from virus infection by inducing an antiviral state and modulating the immune response (Durbin et al, 2000). There are two distinct families of IFNs, IFN-α, -β (type I), and IFN-γ (type II). IFN-α is largely characterized as inducing a "cell-autonomous antiviral state" (Hober et al, 1999). IFN-α is produced by influenza infected epithelial cells and by monocytes/macrophages. Using IFN-α, -β receptor knockout

mice Julkuner et al. illustrated the importance of IFN Type I system in antiviral defense against influenza by showing the increased susceptibility to the infection in the knockout mice (Julkuner et al, 2001).

IFN-γ is known as an immunomodulator (Durbin et al., 2000). IFN-γ is a cytokine produced by NK cells, CD4+ Th1 cells, and a subset of CD8+ T cells. It is a major defense against viruses. It can block viral replication or even lead to elimination of virus by promoting killing of infected cells. It stimulates the cellular response by inhibiting the ability of some microbes to multiply, and it promotes apoptosis of infected cells (Bot et al., 1998). IFN-γ can upregulate the presentation of viral peptides in MHC class I molecules of infected cells, thus allowing killing of virally infected cells by CTLs (Graham et al., 1996). Other effects of IFN-γ include upregulation of antigen processing, presentation of MHC class I and II, and molecules that regulate priming, recruitment, and death of activated T lymphocytes. IFN-γ can also increase the function of NK cells and macrophages (Bot et al, 1998).

Influenza and TNF-α

Tumor necrosis factor alpha (TNF-α) has multiple functions. It is important in inflammation, increases MHC I display on target cells, and boosts CTL killing. In response to influenza, TNF-α up-regulates adhesion molecules on endothelial cells of post-capillary venules causing cell extravasation into the lung (Hussel et al., 2001). Studies have found that TNF-α may play a role in early neutrophil and eosinophil recruitment by increasing chemokine production (McKinney et al., 2003). Other studies show that although TNF-α is increased during influenza infection and that anti-TNF-α administration may actually improve mouse survival. These studies suggest that TNF can exacerbate influenza infection by increasing

the activation of inflammatory cells. Thus, decreased amounts of TNF could lead to a reduction in lung damage to otherwise healthy areas of the lung caused by over-activation of inflammatory cells (Hussel et al., 2001).

Influenza and IL-2

Studies have shown that baseline concentrations of the T helper 1 cytokine IL-2 decline with age. Aged mice have been shown to have decreased CTL activity against virally infected targets, reduced proliferative responses, lower interleukin-2 production, defective antigen presentation, and a decreased number of splenic CTL precursor cells (Bender et al, 1993). Naïve cells have a higher production of IL-2, which is critical in T cell proliferation. The decline in IL-2 could be due to the shift from naïve to memory cells in an aged population. One study reported a diminished proliferative response and decreased IL-2 production in cell cultures from old mice compared to those from young mice following stimulation with influenza virus. Furthermore, they found that addition of exogenous IL-2 increased the proliferative response of T cells and immunity to influenza (Effros et al., 1983).

Influenza and IL-12

There is evidence for diminished natural killer cell activity in aged individuals compared to young (Fiatarone, 1987). Endogenous IL-12 contributes to early NK cell-dependent IFN-γ production (Kostense et al., 1998). IL-12 contributes to the inhibition of early virus replication but is not required for virus clearance. IL-12 is important to the activation of CTLs and to the development of type I T helper cells (Monteiro et al., 1998). IL-12 is a strong inducer of IFN-γ, which aids in the differentiation of T helper 1 cells. It induces the maturation of Th1 cells and therefore, is important in augmenting cell-mediated immunity (Shearer et al., 1997). In influenza infection, IL-12 contributes to the early

development and activation of the innate immune responses (Monteiro et al., 1998). However, there is conflicting data on the role of IL-12 in influenza. It is possible that IL-12 plays a role in attenuating the immune response. In a study by Kostense et al. (1998) IL-12 administration in mice was found to enhance Th1 activity but IL-12 delayed recovery from influenza. This delay in recovery was due to reduced viral clearance (higher viral titers). Thus, IL-12 may have both positive and negative features in response to a viral challenge.

Costimulatory molecules on APC and influenza infection

Dendritic cells are considered to be powerful antigen-presenting cells (APCs). APCs are critical to an effective immune response. Dendritic cells destroy pathogens, but more importantly they help to mount an adaptive immune response (Janeway et al., 2001). They take up antigenic molecules, become activated, and become APCs. Dendritic cells are the only APCs capable of priming the lymph node-based naïve T cells (Janeway et al., 2001). APCs migrate from peripheral sites of pathogen entry to the T cell areas of the lymph node where they provide naïve T cells with Ag-specific and costimulatory signals. APCs tell the immune system the molecular identity and pathogenic potential of an invader (Mailliard et al., 2003). Activated dendritic cells secrete cytokines that can affect both adaptive and innate immunity. Because dendritic cells are the most powerful APC, their function is critical to cellular immunity against influenza (Mailliard et al., 2003).

In order to proliferate and differentiate in response to a viral challenge, T cells need two signals. The MHC bound with antigenic peptide interacting with the TCR gives one signal. The other signal is the CD28 costimulatory molecule interacting with B7.1 (CD80) or B7.2 (CD86) on APCs (Janeway et al., 2001). Studies have shown that B7.1 is necessary for T cell activation in the lungs (Lumsden et al., 2000). In fact, the T cells stimulatory ability of

mouse lung dendritic cells *(in vitro)* is solely dependent on B7.1 expression whereas B7.2 has a secondary role (Lumsden et al. 2000).

Many immune parameters are important in the prevention of, and recovery from influenza. However, many of the critical immune mediators are attenuated in aged animals. Thus, it is important to find an intervention that can increase immune responsiveness and improve resistance to influenza infection. The purpose of this research was to determine the effect of moderate exercise on both resistance and immune responsiveness to influenza infection in an aged population. We used a well-characterized mouse model of influenza infection to determine how exercise affected viral lung titers, lung lesion formation, anti-viral cytokine production (IL-2, IL-10, IL-12, IFN-γ, TNF-α), dendritic and macrophage populations and function.

The hypothesis of this study was that exercise would enhance resistance to infection. Specifically, exercise would decrease lung viral titer, lessen the histopathology by decreasing the number and severity of lesions, and would positively affect immunity in regards to viral induction of the cytokines TNF-α, IL-2, IL-10, IL-12, IFN-γ, and IFN-α. We also hypothesize that exercise will up-regulate B7.1 and B7.2 molecule on splenic cells.

Materials and Methods

Animals

Male BALB/c (n-40) were purchased from Jackson laboratories and allowed to age to 21-23 months. During that time they acclimated to their environment and a 12-hour light: 12-hour dark cycle. Following acclimation, aged mice were randomly assigned to an exercise (n=17) or non-exercise (n=17) group. A third group consisted of non-exercise uninfected control mice (n=4).

Exercise training

Mice were trained on a treadmill for 10 weeks. Exercise training started at a moderate pace of 6 meters per minute and gradually increased to 14-17 meters per minute for 45 minutes five times a week during the light phase. The non-exercised and control mice were housed in the same place as the exercised mice and exposed to the same noise of the treadmill.

Virus

A/PR/34/8 (H1N1) influenza virus was grown in chicken eggs. After harvest the hemagglutinin titer of the virus was determined. Virus stocks contained 512 haemagglutinating units (HAU) per 0.05 ml. The virus was stored in aliquots at –70°C. Prior to use, the virus was thawed in ice and kept on ice.

Inoculation

Within 4 hours of exercise, the mice were lightly anesthetized with CO_2 and infected with approximately 20 HAU of influenza in a volume of 40 μl per mouse via the intranasal route. The mice were held for approximately 30 seconds to ensure the virus reached the respiratory tract. The intranasal route was chosen to mimic the typical route of entry of

influenza infection. After infection, all mice were returned to their original cages with their same littermates and symptoms were monitored and recorded daily. Exercised mice were not exercised after inoculation. One exercised mouse died 5 days post infection. At necropsy, a large tumor unrelated to the influenza infection was observed on the lung.

Collection of cells and tissue

Mice were euthanized using a CO_2 overdose 6 and 10 days post infection. Blood was immediately collected via heart puncture. The thoracic cavity was opened exposing the trachea, ribcage and diaphragm. The diaphragm and ribcage were cut away to expose the lung. One lobe on the left side of the mouse was clamped off using a small hemostat. This lobe was removed and analyzed for influenza viral titer. The remaining lung was inflated with formalin though the trachea using a syringe.

The spleen was removed, placed in a stomacher and crushed into a single cell suspension in AIM-V medium. Mediastinal lymph nodes were removed and crushed using frosted microscope slides into a single cell suspension in AIM-V medium.

Histopathology

The lung lobes were inflated and fixed with formalin using a syringe (with the exception of one lobe that was used for viral titer analysis). After inflation, the lungs were immersed in formalin. After fixation in formalin for a least one day, each lung lobe was trimmed, and embedded in paraffin, and processed into histologic sections by routine methods in the Iowa State University Veterinary Pathology Laboratory. Histopatholologic evaluation was conducted by a pathologist blinded to the identity of the groups. The number of lobes (out of 4) with lesions were recorded. Lesions were graded by estimating the percent of involvement for each lobe section. A score of 0= no lesions; 1= 0-25%; 2= 26-

50%; 3= 51-75%; 4= 76-100%. The average score for the 4 lobes was calculated for each mouse.

Cell cultures

The splenocytes were drawn up into a pipet and passed through a nylon screen filter into a 15 ml centrifuge tube. Hanks Balanced Salt Solution (Sigma Chemical, St. Louis, MO) was used to rinse the stomacher bag and the additional cells were also passed through the filter into the centrifuge tube. The cells were centrifuged at a speed of 1000 rpm for 5 minutes and decanted. Approximately 2-3 ml of ice-cold ammonium chloride was added to lyse red blood cells and the tube was allowed to sit for 2 minutes followed by the addition of 10 ml of RPMI. The cells were centrifuged for 5 minutes at 1000 rpm and washed two times with Hanks Balanced Salt Solution (HBSS). After the final wash, the cells were resuspended in 10 ml AIM-V media (Life Technologies, Grand Island NY). The cells were counted using a Coulter counter (Beckman-Coulter, Hialeah, FL) and the concentration was adjusted to 3 x 10^6 cells/ml with AIM-V.

Cells were incubated for various times with and without the addition of influenza virus. One-ml aliquots of cells were placed in a 24-well tissue culture plates. In cultures with virus, the A/PR/34/8 Influenza virus stock was diluted 1: 51.2 and 50 µl of virus (10 HA units/well) was added to cell cultures before being placed in the incubator. The cell culture supernatant collection was as follows: *IL-2*: 24, 48, 72hr; *IFN-γ*: 48, 72, 96hr; *IL-10*: 48, 72, 96hr; *TNF-α*: 24, 48hr; *IL-12*: 24, 48, 72hr. *IFN-α:* 24hr. Tissue culture plates were placed in an incubator at 37°C and 5% CO_2 for the designated incubation time.

Optimum virus culture conditions were not obtained. Cells incubated with virus did not contain any greater cytokine amounts then the non-virus culture cells. Therefore, the

results shown are those of cells incubated in media only. Thus, data for cytokines reflects those produced in response to *in vivo* infection.

Flow cytometry

Splenocytes (1ml) were aliquoted into 24-well plates with culture media. Splenocytes were incubated with live virus (10 HA units/ml) for 24 hours. Five hundred μl of cells and 10 HA units/well of A/PR/34/8 virus at were added to 2 sterile 12*75mm polypropylene tubes per mouse. CD11c and Mac-1 (CD11b) (CD80 and CD86 were evaluated). A standard flow protocol was followed. Briefly, the cells were washed with cold PBS-0.1% azide and stained for 30 minutes with the following antibodies: anti-CD11c (fluorescein isothiocyanate (FITC)), anti-CD80 (B7.1) (phycoerythrin (PE)), anti-CD86 (B7.2) (PE), and Mac-1 (CDllb) (FITC) (BD Pharmingen, San Diego, CA). The appropriate isotypes were analyzed as well. The results of the Mac1 and CD11c are not shown due to problems gating these populations during flow cytometry analysis.

Mediastinal Lymph nodes

Mediastinal lymph nodes were collected and put into petri dishes with 1 ml of HBSS. The nodes were gently ground using frosted microscope slides and rinsed well with HBSS. The cells were passed through a sterile filter screen, placed into a 15 ml tube, and centrifuged for 5 minute at 1000 rpm. The cells were washed once with HBSS and resuspended in 500 μl of AIM-V media. The cells were counted using a hemocytometer and trypan blue and were adjusted to $1x10^6$ cells/ml using AIM-V media. 220ul of cells and 10 HA units/well virus were added to each well in a 96-well plate (for IL-2 and IFN-γ ELISA analysis). The cells were incubated for 48, 72, & 96 hours at 37°C with 5% CO_2. Supernatants were collected and stored at –20°C for analysis by ELISA.

ELISA

Commercially available ELISA kits (BD Pharmingen, San Diego, CA) were used to determined cytokine concentrations in supernatants from splenocytes or lymph node cultures. The cytokines IFN-γ, TNF-α, IL-2, IL-10, IFN-α, and IL-12 were measured.

Virus titer

Viral titer in the lungs was determined by quantitative Polymerase Chain Reaction (PCR).

Statistics

A one-way ANOVA (SPSS software) was used to compare differences in the immune parameters (viral titer, lung lesion score, cytokines, CD80/86) between the exercise and control group. Day 6 and 10 were compared in separate analyses. In the analyses that contained more that one in vitro measure (cytokines at multiple time points in vitro), a one way mixed ANOVA (group * time) was used. A one-way ANOVA was also used to compare difference in immune responses between the uninfected control mice and the infected mice.

Results

Exercise decreased lung pathology and influenza viral titer

Histopathology

Lung lesions were graded by estimated percent of lung involvement (graded for each lobe and average of all four lobes calculated for each mouse). No lesions were observed in any of the control uninfected non-exercised mice. In the group of mice that were euthanized at 6 days post infection, six of nine non-exercised mice developed lung lesions (five of affected mice had 4/4 lobes affected and an average lobe score of 3.25). Only 3 of 9 exercised developed lesions with two of those three showing 4/4 lobes affected (average lobe score 3.125) (Figure 1). However, the total lesion score (lesions averaged for all mice) was not significantly different between non-exercise and exercise mice at day 6.

In the non-exercised mice that were euthanized at 10 days post infection five of ten mice developed lung lesions. Four of five mice had lesions in 4/4 lobes (average lobe score 3.06). The average lesion score was significantly lower in exercise mice compared to non-exercise mice ($p=.05$). Only two of the eight exercised mice euthanized at day 10-post infection had lesions with 4/4 lobes affected, and with an average lobe score of 2.0 (Figure 1). Thus, fewer exercised mice developed lesions. Exercised mice that did have lesions had less intensive lung involvement.

Viral Loads

Virus was not detected in the lungs of uninfected mice. At day 6 the non-exercised mice had higher viral titers ($p= .006$) than the exercised mice (Figure 2a). At day 10 there was no significant difference between the exercise and non-exercised mice (Figure 2b).

Splenocyte Cytokines

Splenocytes from uninfected mice secreted virtually no TNF-α whereas influenza infection resulted in greater production of TNF-α (p=.09) (Figure 3). There was no difference between the amount of TNF-α produced by cells incubated for 24 hours in exercised mice compared to non-exercised mice at day 6 and 10 days post-infection. However, in a comparison between day 6 and day 10, higher amounts of TNF-α were produced at day 10 (p-value < .001) (Figure 4).

IL-2 concentrations ranged from very low to undetectable in the uninfected mice. Influenza infection appeared to increase the production of IL-2 (p=.1) (Figure 5). Non-exercised mice at day 6 produced little to no IL-2 whereas the exercised mice produced significantly more IL-2 (p = .01). At day 10, there was a statistical trend suggesting that splenocytes from exercise mice produced higher amounts of IL-2 the than the non-exercise mice (p = .09) (Figure 6).

There was a trend toward as greater secretion of IL-10 in infected mice compared to uninfected mice (p = .08) at 72 hours of in vitro culture (Figure 7). The cells of the exercise mice secreted significantly higher amounts of IL-10 than the non-exercised group, at day 6 post infection (p = .04) (Figure 8). At day 10 post-infection the amount of IL-10 produced was not significantly different between exercise and non-exercise.

Figure 9 shows a slight trend toward a difference in the amount of IFN-γ produced between the uninfected and infected mice (p=.1), with the uninfected mice having low to no IFN-γ production. At day 6 and 10, there was no significant difference in the amount of IFN-

γ produced by the splenocytes from exercised mice as compared to non-exercise mice (Figure 10).

Figure 11 shows no significant difference in IL-12 production between uninfected and infected mice at both 24 and 48 hours in vitro culture. There was no difference between exercise and non-exercise mice with respect to the amount of IL-12 produced at day 6 or 10 post-infection (Figure 12 b).

Splenocytes from infected mice produced significantly more IFN-α than splenocytes from uninfected mice ($p = .06$) (Figure 13). The cells from the exercise group produced significantly greater amounts of IFN-α than the control group ($p = .05$) (Figure 14).

Mediastinal Lymph Node Cytokines

Infection tended to result in higher amounts of IFN-γ in the lymph nodes of infected mice compared to those of uninfected mice ($p = .09$) (Figure 15). At day 6 there was a trend toward greater IFN-γ produced by lymph nodes cells from the exercise group compared to the control group ($p = .08$) (Figure 16).

Figure 17 and 18 show the amount of IL-2 produced in the mediastinal lymph node. There was no significant difference between infected and uninfected mice, or between the exercise and non-exercise mice.

Flow Cytometry

Influenza infection showed a trend towards increased CD86 expression ($p = .09$) (Figure 19). At day 6, the expression of CD86 expression appeared to be greater in the exercise group as compared to the controls ($p= .07$), but there was no difference at day 10. There was no significant difference in CD80 between infected and uninfected mice or between the control and exercise mice (Figure 21 & 22).

Figure Legend

Figure 1. Average lesions score of influenza infected mice. Score is calculated from all mice from each group whether lesions present or not. Exercise mice have a lower lesion scores at day 6 post-infection ($p = .05$).

Figure 2. Lung viral titers 6 days post influenza infection in infected non-exercised mice and infected exercise mice. At 6 days post infection exercise mice have reduced lung viral titers compared to controls ($p = .006$) (Figure 2a). There is no significant difference between viral titers at 10 days post infection (Figure 2b).

Figure 3. Amount of TNF-α produced by splenocytes in infected and uninfected aged mice. Data from day 6 and 10 were combined. Infected mice tended to produced more TNF-α than uninfected mice ($p = .09$). The time points shown are 24 and 48 hours of *in vitro* culture.

Figure 4. Amount of TNF-α produced by splenocytes in infected non-exercise and infected exercised aged mice. Day 6 and 10 were analyzed separately. The time point shown is 24 hours *in vitro* culture (Figure 4). No difference between exercise and non-exercise was found.

Figure 5. Amount of IL-2 produced by splenocytes in infected and uninfected aged mice. Data from day 6 and 10 were combined. The time points shown are 24 and 48 hours of *in vitro* culture. IL-2 tended to be higher in infected mice.

Figure 6. Amount of IL-2 produced by splenocytes in infected non-exercise and infected exercised aged mice. Data from day 6 and 10 were analyzed separately. The time point shown is 24 hours *in vitro* culture. IL-2 production was significantly higher in exercise mice compared to non-exercise at 6 days post-infection ($p = .01$), whereas a

trend toward a higher production of IL-2 by exercise mice was observed at day 10 post-infection (p= .09).

Figure 7. Amount of IL-10 produced by splenocytes in infected and uninfected aged mice. Data from day 6 and 10 were combined. The time points shown are 72 and 96 hours. At 72 hours infected mice tended to produce greater IL-10 than uninfected mice (p= .08),

Figure 8. Amount of IL-10 produced by splenocytes in infected non-exercise and infected exercised aged mice. Data from day 6 and 10 were analyzed separately. The time point shown is 72 hours *in vitro* culture. IL-10 produced by exercise mice was significantly greater than non-exercise mice at day 6 post-infection (p= .04). No difference between groups was found at day 10.

Figure 9. Amount of IFN-γ produced by splenocytes in infected and uninfected aged mice. Data from day 6 and 10 were combined. The time points shown are 72 and 96 hours.

Figure 10. Amount of IFN-γ produced by splenocytes in infected non-exercise and infected exercised aged mice. Data from day 6 and 10 were analyzed separately. The time point shown is 72 hours *in vitro* culture. No difference was found between the groups.

Figure 11. Amount of IL-12 produced by splenocytes in infected and uninfected aged mice. Data from day 6 and 10 were combined. The time points shown are 24 and 48 hours. There was no difference between the groups.

Figure 12. Amount of IL-12 produced by splenocytes in infected non-exercise and infected exercised aged mice. Data from day 6 and 10 were analyzed separately.

The time point shown is 24 hours *in vitro* culture. No difference was found between the groups.

Figure 13. Amount of IFN-α produced by splenocytes in infected and uninfected aged mice 6 days post infection. The cells were cultured in vitro for 24 hours *in vitro* culture. Infected mice tended to produce more IFN-α than uninfected mice (p= .06).

Figure 14. Amount of IFN-α produced by splenocytes in infected non-exercise and infected exercised aged mice. Only 6 days post-infection was evaluated. The time point shown is 24 hours *in vitro* culture. Exercise mice produced significantly more IFN-α than non-exercise mice (p= .05).

Figure 15. Amount of IFN-γ produced by mediastinal lymph node cells of influenza infected and uninfected aged mice. Data from day 6 and 10 were combined. The cells were cultured in vitro for 72 hours. Infected mice tended to produced greater IFN-γ than uninfected (p= .09).

Figure 16. Amount of IFN-γ produced by the mediastinal lymph node cells in infected non-exercise and infected exercised aged mice. Data from day 6 and 10 were analyzed separately. The time point shown is 72 *in vitro* culture. At day 6 post-infection IFN-γ production tended to be higher in exercise than non-exercise mice (p= .08). There was no difference between groups at day 10.

Figure 17. Amount of IL-2 produced by mediastinal lymph node cells of influenza infected and uninfected aged mice. Data from day 6 and 10 were combined. The cells were cultured *in vitro* for 48 hours. No difference was found between groups.

Figure 18. Amount of IL-2 produced by the mediastinal lymph node cells in infected non-exercise and infected exercised aged mice. Analyzed 6 and 10 days post infection separately. The time point shown is 48 *in vitro* culture. No difference was found between groups.

Figure 19. The percent of splenic cells expressing CD86. Infected mice tended to demonstrate greater expression of CD86 then uninfected ($p = .09$).

Figure 20. Percent of splenocytes expressing CD86 in infected non-exercise and infected exercise. At day 6, CD86 expression tends to be greater in exercise mice compared to non-exercise mice ($p = .07$), whereas there is no difference at day 10.

Figure 21. The percent of splenic cells expressing CD80. Uninfected mice were compared to infected mice with respect to CD80 expression. There was no difference between the groups.

Figure 22. Percent of splenocytes expressing CD86 in infected non-exercise and infected exercise. Data from day 6 and 10 were analyzed separately. There was no difference between the groups.

Graphs

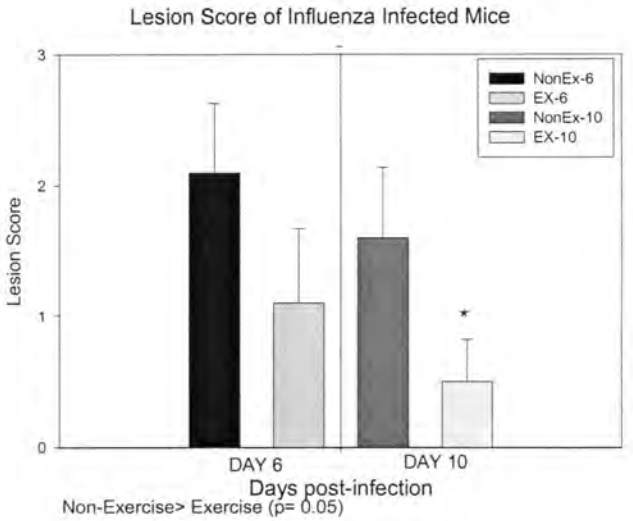


Figure 1

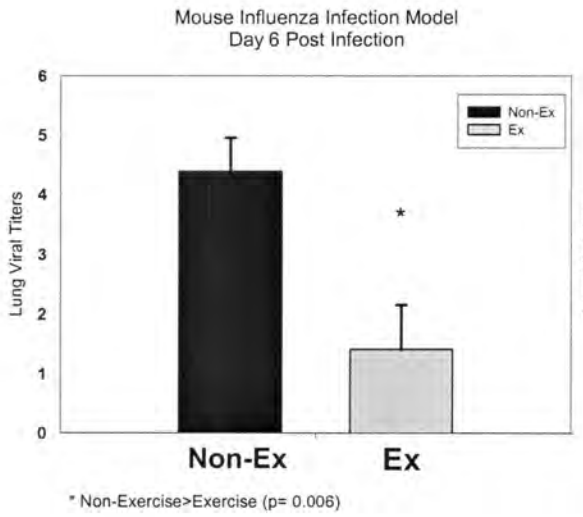


Figure 2a

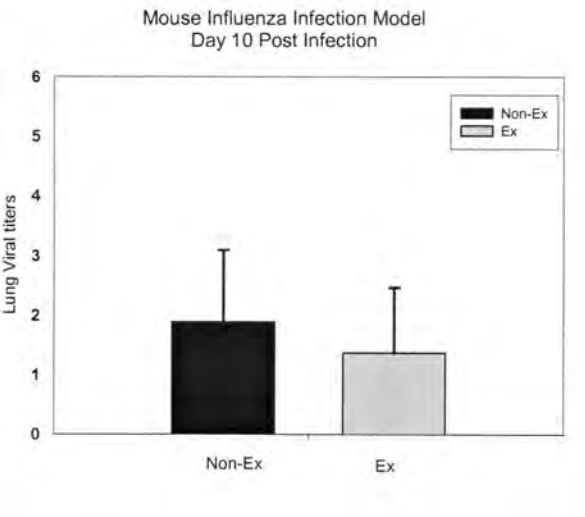
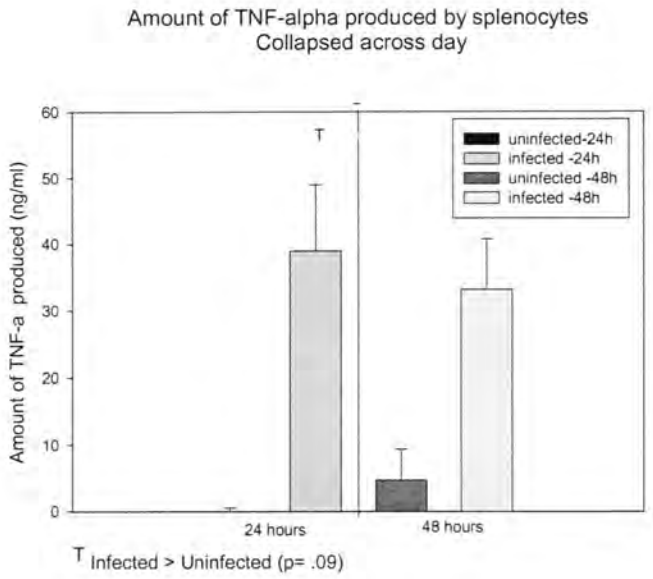


Figure 2b



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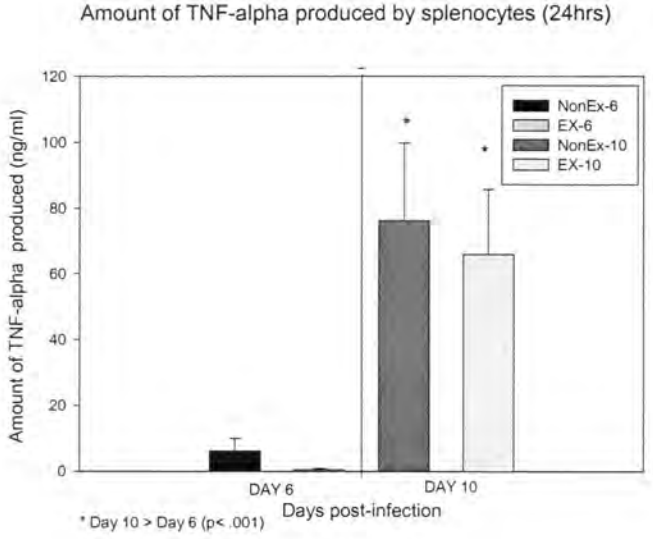

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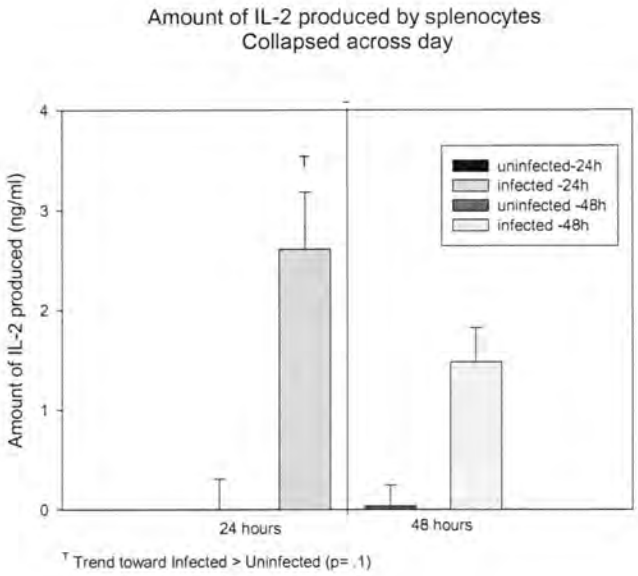

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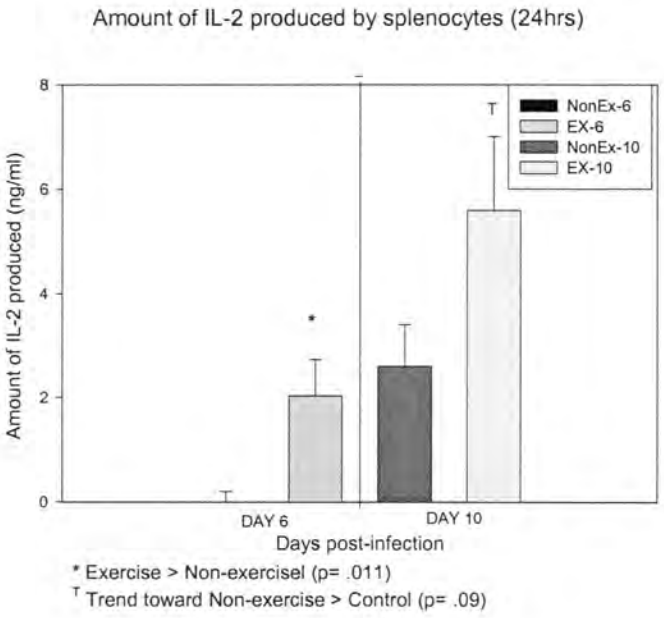

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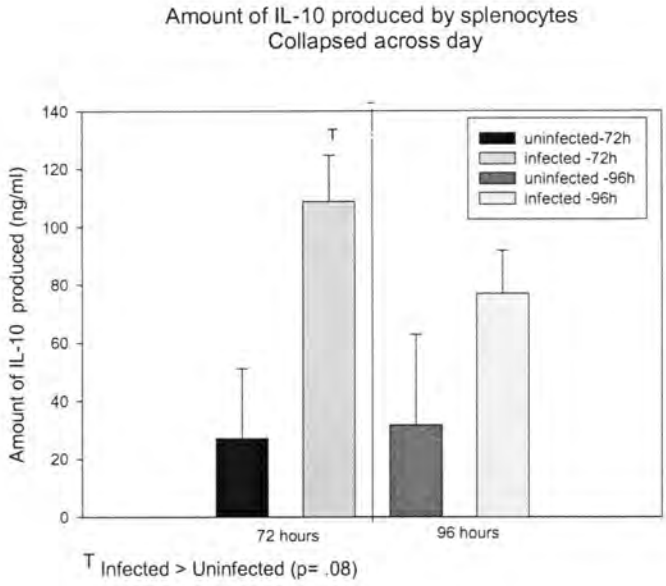

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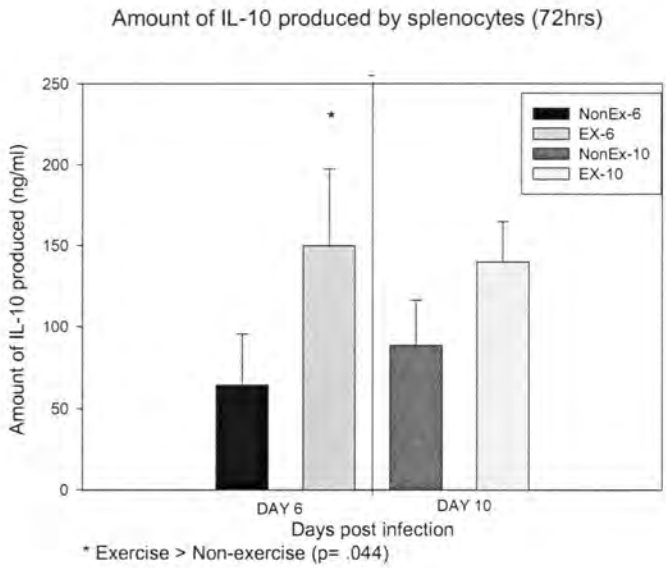

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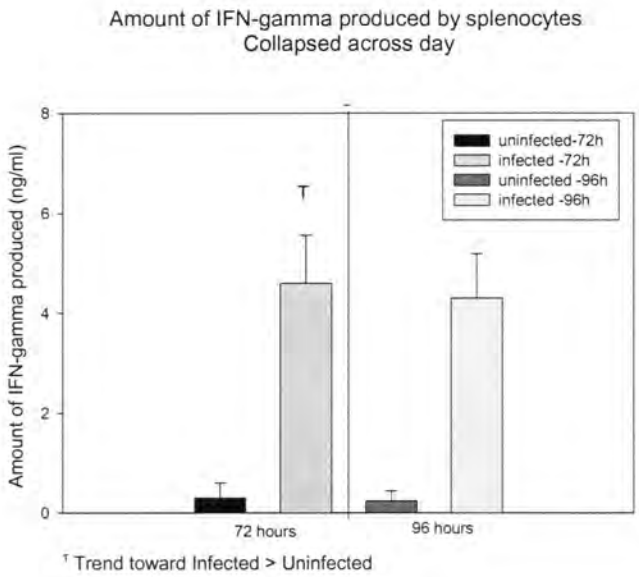

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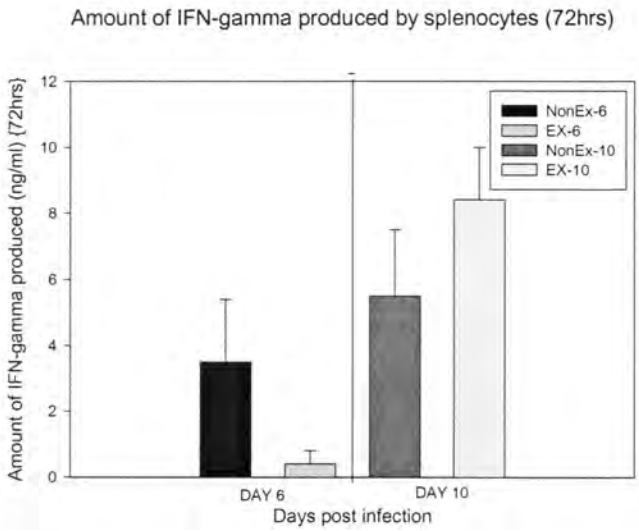

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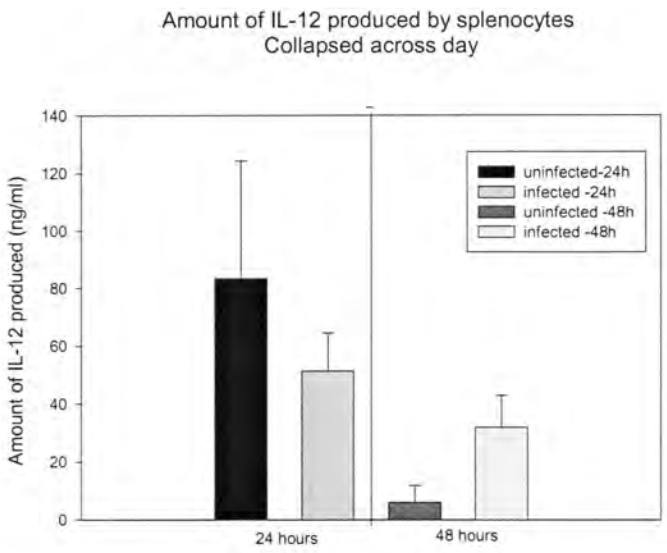

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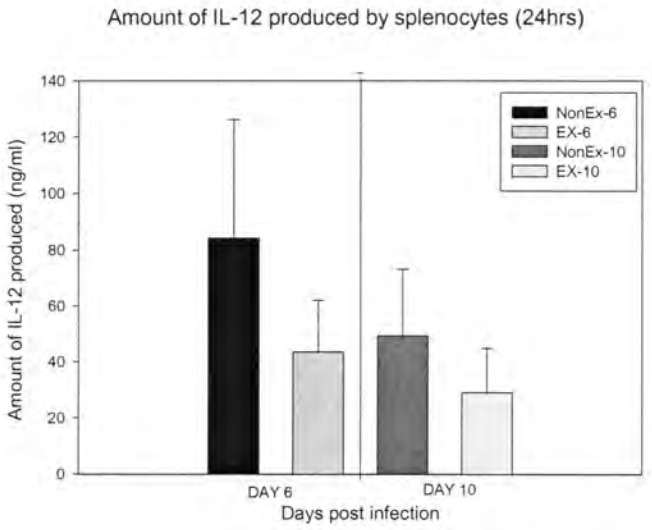

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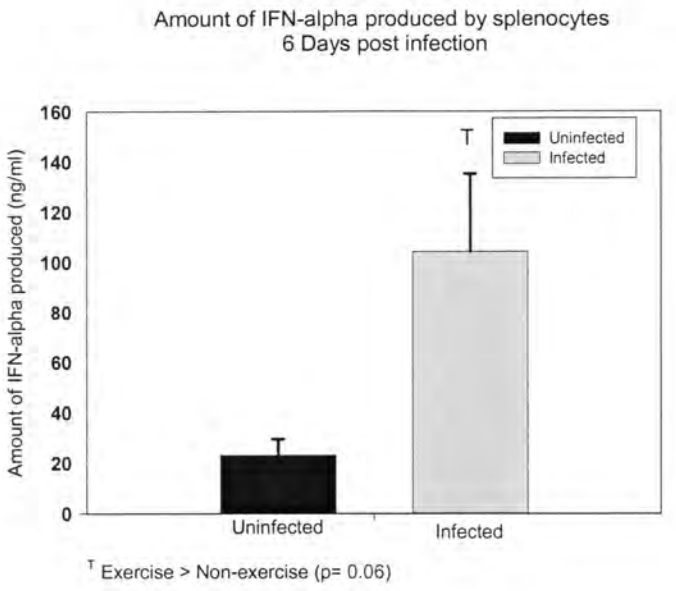

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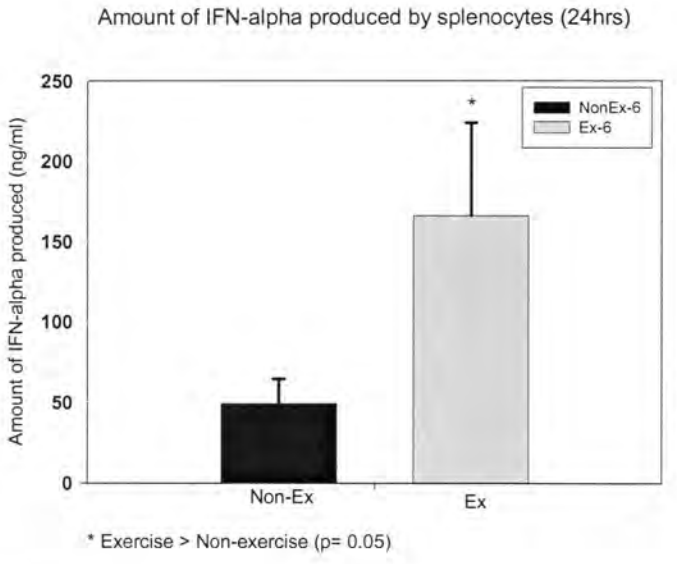

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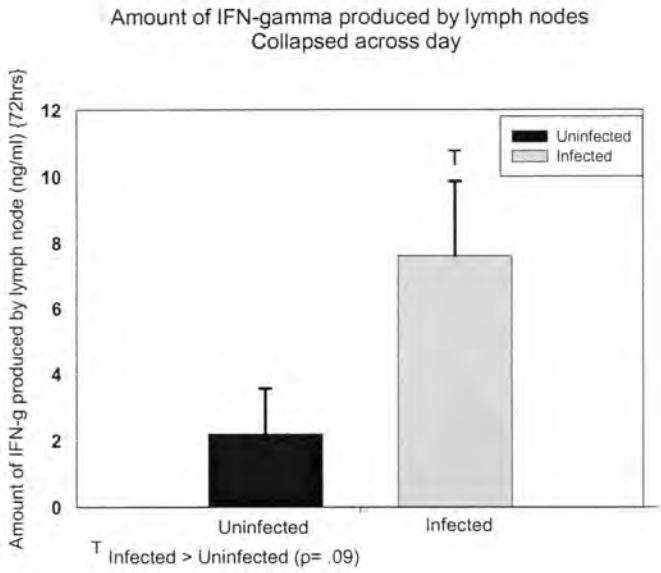

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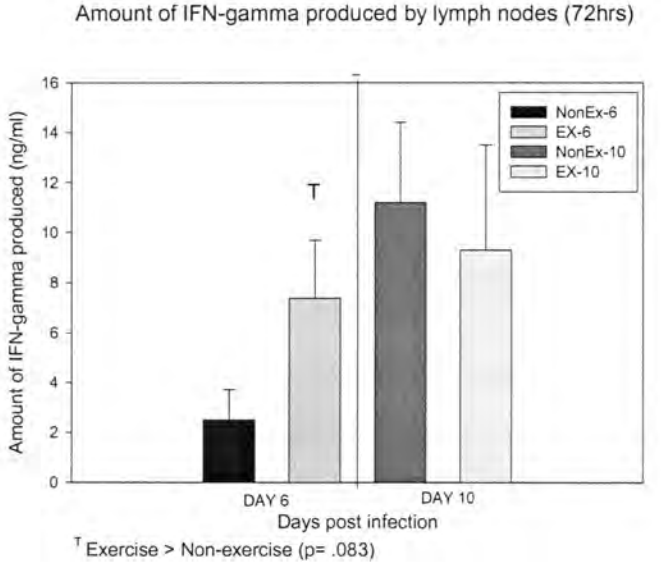

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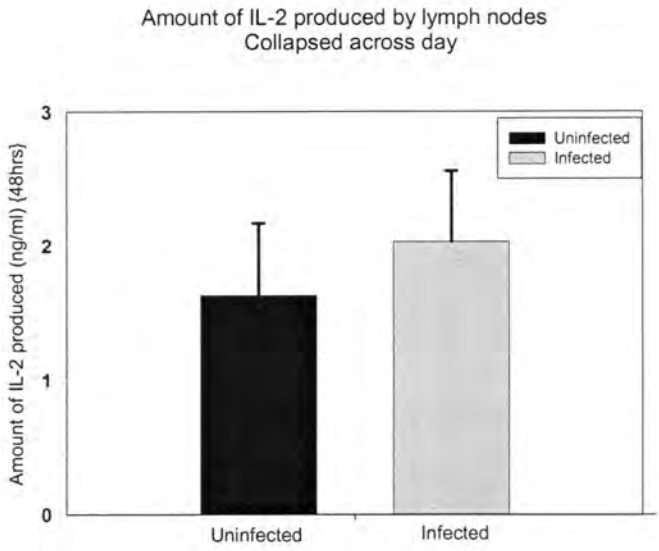

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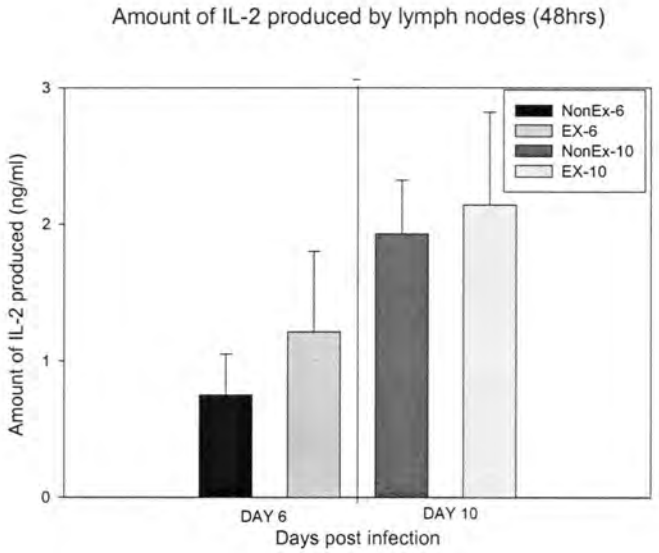

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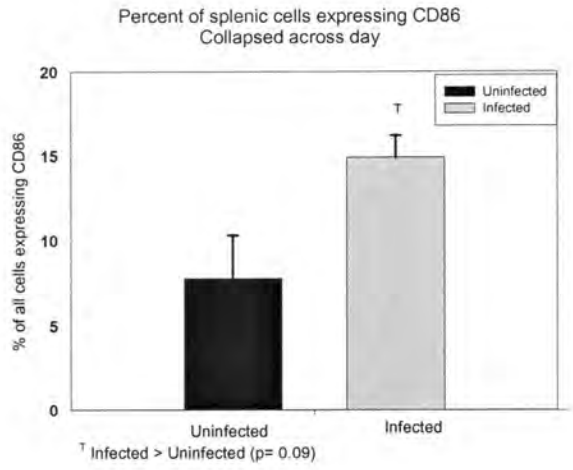

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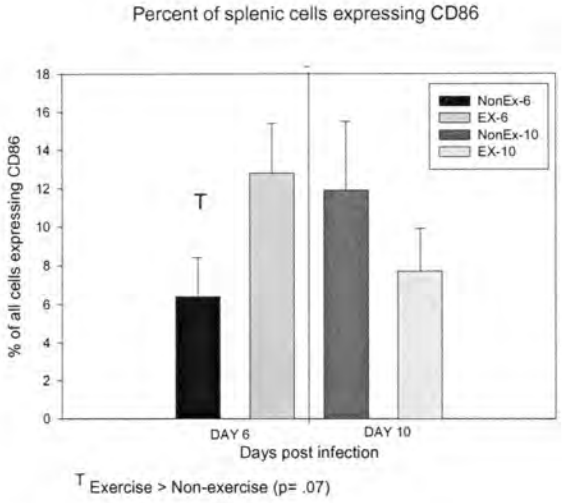

Figure 20

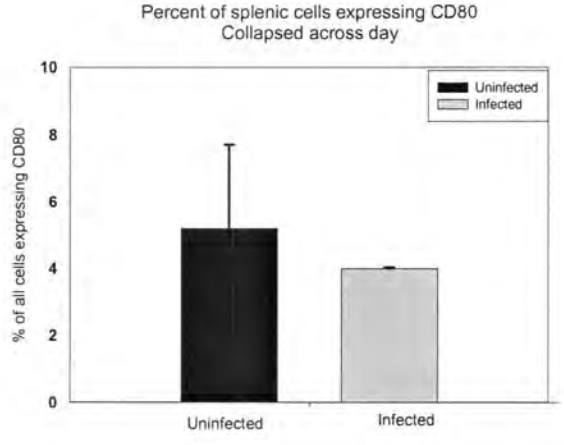

Figure 21

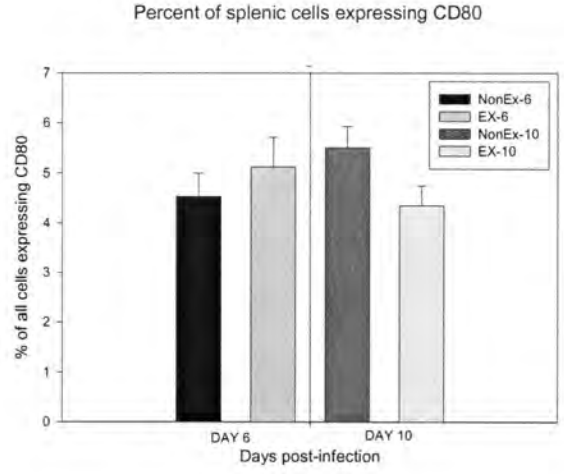

Figure 22

Discussion

As we expected, infection of mice with the influenza virus led to a histopathological picture characterized by an accumulation of inflammatory cells and destruction of respiratory epithelium (Bender, 1995). Our results showed that exercise mice developed fewer lesions and the lesions that developed were less severe than in the control mice. These findings suggest that moderate exercise training has beneficial effects in terms of reducing the pathology associated with influenza infection. We also observed that exercise reduced the viral titer at day 6 post-infection. Although it is possible that viral titers were also diminished in the exercise group at earlier time points (prior to day 6), we did not evaluate earlier time points in this experiment. Our preliminary studies suggested that lung viral titers appeared to peak near day 3-4 post-infection and begin to decline by day 6 post-infection. To our knowledge, this is the first study to demonstrate that exercise training in an aged population can reduce the severity of viral infection.

We also observed that exercise training affected both nonspecific and specific immune defenses during viral infection. IFN-α is an important innate defense against viral infection, and we found that IFN-α was significantly increased with exercise. This may be one reason for the decline in lung viral titer, although we will need to measure IFNα at earlier time points in the lung to confirm this.

There was also an increase in splenocyte TNF-α production in response to influenza infection, particularly at day 10. To our knowledge, this delayed increase of TNF-α is the spleen in response to influenza infection has not previously been shown. TNF-α is critical to cell recruitment and up-regulates adhesion molecules on endothelial cells of venules, leading

to cell influxes into the lung. However, we found that there was no significant difference between the exercise and non-exercise groups at the time points measured.

IFN-γ is an inflammatory cytokine that helps to recruit immune cells to the site of infection. Baugarth and Kelso (1996) have shown that anti-IFN-γ antibody given to influenza infected mice drastically reduced the cells that infiltrated the lungs. Therefore, it seems possible that higher amounts of virally induced pulmonary IFN-γ are related to increased inflammation. However we did not measure pulmonary IFN-γ and therefore, we cannot determine whether exercise has an effect on lung IFN-γ. IFN-γ is an TH1 cytokine that promotes up-regulation of CTL function. In the draining lymph node to the infection IFN-γ tended to increase in the exercise mice at day 6 suggesting improved CTL function early in the infection.

A strong immune response to influenza requires a "complex interplay" between CTLs, antibody secreting B cells, and cytokine secreting CD4 cells (Gerhard, 2001). IL-2 is a immune system messenger which is involved in the proliferation of CD4 cells. CD4 cells contribute to immune responses at the site of infection by secreting cytokines (Brown et al, 2004). Depletions studies have shown that CD4 cells are also required to provide help for CTLs and B cells (Mozdzanowska et al, 2000). Exercise training significantly increased IL-2 production 6 days post infection. This may suggest that the increased IL-2 production in exercise mice enhanced antiviral defenses (CTLs, B cells, and CD4 proliferation).

We did not find a difference in IL-12 production between exercise and non-exercised mice. Previous studies demonstrated potential adverse consequences of IL-12 administration. Mice injected with IL-12 were found to have delayed viral clearance,

increased mortality, and higher amounts of pulmonary inflammation. The researcher suggested this was due to higher amounts of induced pulmonary IFN-γ correlated with the IL-12 treatment (Kostense 1998). High amounts of IL-12 could lead to increased disease severity by activating bystander inflammatory cells, thus damaging otherwise healthy areas of the lung.

Other studies have shown that IL-12 is important in early activation of the immune response in influenza infection through early induction of IFNγ, subsequently leading to an inhibition of early virus replication and the activation of CTL's. It has been suggested that, as the immune response progresses, it becomes more antigen driven and IL-12 is no longer needed. Thus, the mice are able to recover from influenza virus without IL-12 (Montessori 1997). It may be helpful in future studies to examine IL-12 production early in infection perhaps in the lungs and determine whether exercise may influence IL-12 potentially resulting in early activation of the immune response.

The CD28 costimulatory pathway is crucial for T cell activation. A study by Lumsden et al (2000) found that blocking the interaction of CD28 with CD80 and CD86 in influenza infected mice reduced virus-specific cytotoxicity, IFN-γ production, virus specific CD8+ cells, and resulted in lower virus-specific antibody titers. In this experiment the mice were still able to clear the virus, but viral clearance was delayed in comparison with controls. The researchers also found that blocking only the CD80 ligand did not affect immunity as long as the CD86 ligand was still able to bind to CD28 (Lumsden et al., 2000). This could explain why we observed no difference between infected and uninfected mice with respect to the expression of CD80. We found that the expression of CD86 cell populations tended to be greater in infected mice compared to uninfected mice. Also, at 6 days post-infection, there

was an increase of the percent of cells expressing CD86 in the exercise trained mice compared to non exercise mice. Higher percent of CD86+ cells may be suggestive of a greater number of antigen presenting cells, potentially leading to better antigen presentation. In addition, higher CD86 expression could suggest that there is an upregulation of specific T cells.

The mechanisms by which exercise alters immune responses are not clear. However, it is known that physical activity can influence neuroendocrine levels in the central nervous system and in peripheral blood. Changes in immune function that occur in response to exercise may be regulated by various neurohormones. Neuropeptides altered by exercise that may affect the immune system include sertonin, substance P, and vasoactive intestinal peptide (Pedersen et al., 2000). β-endorphin is an opioid peptide released during exercise that can also affect immune function. Studies have show that β-endorphin enhances NK cell activity and IFN production in vitro. It has also been shown to regulate macrophage, T and B cell functioning (Jonsdottir et al., 2000). It is possible that the release of β-endorphin during exercise acts to enhance immune function.

β-adrenoreceptors are present on many immune cells such as T and B-lymphocytes, neutrophils, macrophages, and NK cells. Cells respond to catecholamines via β-adrenergic receptor activation. Catecholamines increase during exercise and have numerous effects on immune cell function (Madden et al., 2000). In a recent three-week mouse exercise training study by Itoh et al. found that exercise down-regulates β-adrenergic receptor expression resulting in enhanced immune responsiveness. This decline β-adrenergic receptor expression improved IL-12 induced type 1 helper T cell mediated immune responses (Itoh et al., 2004).

Overall, we observed beneficial immunological responses with exercise training that may have led to the reduction in viral burden and inflammatory pathology. We tended to find an up-regulation of cytokine secretion early in the infection (day 6) which may have improved resistance to the infection and enhanced viral clearance. This increase was observed both in the draining lymph nodes and the spleen. Also, APC function may have been improved by exercise, as suggested by the up-regulation of CD86 expression. In many instances we observed enhanced immune response to influenza in the exercise mice at day 6, but not at day 10. This suggests that exercise training may shift the kinetics (timing) of the immune response by up-regulating immune defenses more rapidly.

Therefore, our results suggest that exercise may be an effective method of improving resistance to viral infection in the aged, and numerous immune responses (both innate and adaptive) appear to be enhanced by exercise.

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